# IRRADIATED OSTEOINDUCTIVE EXTRACELLULAR
# MATERIAL

## INTRODUCTION

The invention relates to the effect of γ-irradiation on the osteoinductive efficacy of extracellular material obtained from skeletal cells.

The invention relates to the effect of γ-irradiation at various dose levels on the osteoinductive efficacy of lyophilised extracellular material obtained from skeletal cells.

## BACKGROUND TO OSTEOINDUCTION

Extracellular material obtained from skeletal cells may be used in the stimulation of new bone production. The material has a wide range of potential clinical uses in both the dental and orthopaedic markets when used in conjunction with existing medical devices. Extracellular material obtained from skeletal cells has been shown to stimulate the formation of new bone in in-vivo pre-clinical development studies and by in vitro bone formation assay.

The active extracellular material may be derived from cultures of immortalised hypertrophic chondrocyte-like cells. Hypertrophic chondrocyte cells have been shown to play a crucial role in bone formation (Caplan 1988; Olsen et al., 2000). The active extracellular material may be referred to as "Extracellular Matrix" or "ECM".

Replacement of lost bone is the challenge facing orthopaedic surgeons, neurosurgeons, craniofacial surgeons, and periodontists all over the world today.

The surgical repair of lost bone is a problem faced by dentists with patients suffering from periodontal disease, for example. Periodontal disease is one of the most prevalent afflictions, one consequence of which is alveolar bone loss, which in itself is a major disease entity. Presently periodontists and patients work together in treating the symptoms of periodontal disease, and effective techniques that predictably promote the body's natural ability to regenerate lost periodontal tissues (particularly alveolar bone) still need to be developed.

In another field of dentistry, Dental Implantology, a great deal of biomaterial research is being conducted in an attempt to determine factors or substances that can improve the quality of bone to implant contact (osteointegration).

In recent decades a surge in research into understanding bone formation and bone healing has led to the development of various techniques to promote bone healing, or to replace lost bone.

## Endochondral ossification

Endochondral ossification represents the deposition of a bone matrix upon a pre-existing cartilage template and accounts for much of the skeletal formation during embryogenesis and postnatal growth. During the initial phases of this process a region comprising resting or germinal chondrocytes differentiates into a zone of proliferating chondrocytes that then hypertrophies . These hypertrophic chondrocytes become progressively larger, display more mitoses, and are more metabolically active. It is the hypertrophic chondrocytes that lay down the unmineralised and avascular cartilage matrix that is the model for developing bone.

As cell hypertrophy progresses, the pre-existing non-calcifiable and avascular cartilage matrix is transformed to a calcifiable one that is penetrable by blood vessels through angiogenesis . The invading vasculate imports mesenchymal stem cells (MSC's), haemapoietic precursors and osteoclasts . As the osteoclasts degrade the hypertrophic cartilage matrix, mesenchymal stem cells differentiate into primitive marrow cells and osteoblasts; the osteoblasts line the hypertrophic cartilage lacunae in this primary centre of ossification and deposit a bone matrix.

Endochondral ossification is a developmentally regulated process which occurs in a highly co-ordinated temporal and spatial manner, in which there is a sequential recruitment and differentiation of cells which form cartilage, vascular and bone tissues. Such sequence of events relies on the precise coupling of chondrogenesis (cartilage production) with osteogenesis (bone formation).

Hypertrophic chondrocytes play a central role in endochondral bone formation . Chondrocyte hypertrophy is intimately linked to angiogenesis , and when hypertrophy is inhibited, e.g. by parathyroid hormone-related peptide, angiogenesis and subsequent endochondral ossification is blocked , illustrating the major role of hypertrophic chondrocytes in endochondral ossification.

The process of chondrocyte maturation, in conjunction with the establishment of secondary centres of ossification at the outer (epiphyseal) ends of endochondral bone, defines the formation of a growth plate. Growth plates provide bones with longitudinal growth potential until maturity. However, endochondral ossification can be re-initiated during bone healing (e g. fracture repair).

## Bone healing

Bone heals in a unique way compared with other connective tissues. Rather than develop scar tissue, it has the ability to regenerate itself completely . Intact bone is constantly being resorbed and remodelled - a delicate balance coordinated by a rather complex cascade of cellular events.

The majority of fractures heal by secondary fracture healing and that involves a combination of intramembranous and endochondral ossification. The fracture healing sequence involves five discrete stages of healing. This includes an initial stage in which a haematoma is formed and inflammation occurs; a subsequent stage in which cartilage begins to form and angiogenesis develops, and then three successive stages of cartilage calcification, cartilage resorption and bone deposition, and ultimately a more chronic stage of bone remodelling.

It is thought that one of the functions of the haematoma is to be a source of signalling molecules which, in conjuction with others, have the capacity to initiate the cascades of cellular events that are critical to fracture healing.

Perhaps the most important response in fracture healing is that of the periosteum. Here, committed osteoprogenitor cells and uncommitted, undifferentiated mesenchymal stem cells contribute to the process of fracture healing by a recapitulation of embryonic intramembranous ossification and endochondral ossification. It is the ability of factors to stimulate differentiation of osteogenic mesenchymal stem cells, that determines the osteoinductive potential of bone graft substances.

The bone that forms by intramembranous ossification is found further from the site of the fracture, results in the formation of a hard callus, and forms bone directly without first forming cartilage. Two weeks after fracture, cell proliferation declines and hypertrophic chondrocytes become the dominant cell type in the chondroid callus. The resulting endochondral bone is formed adjacent to the fracture site.

Many of the cellular processes that occur during fracture healing parallel those that occur in the growth plate during development, except in fracture healing these processes occur on a temporal rather than a spatial scale.

Fracture healing and bone formation involve a series of distinct cellular responses that are under the control of specific paracrine and autocrine intracellular signalling pathways, it can be viewed as a well orchestrated series of biological events.

## Bone growth factors

Bone is known to be a major source of growth factors . These growth factors have significant effects on bone and cartilage metabolism. This suggests an important role for these growth factors in mediating hormonal responses locally, and a local metabolic reg,ulation of bone metabolism without, necessarily the influence of systemic hormones. They act in a paracrine and autocrine manneer, and are responsible for the highly co-ordinated manner in which bone forms remodels and regenerates after a defect occurs.

Bone derived growth factors stimulate cell replication and contribute to the stimulation of differentiation and metabolic functions of bone cells. They exhibit their effects through binding membrane bound receptors. This leads to a cascade of intracellular events that affect the expression of genes that encode for such metabolic functions as cell division and protein synthesis.

Studies have additionally revealed that cartilage also contains these growth factors suggesting that bone and cartilage interact with each other exchanging growth signals in a paracrine fashion. Hypertrophic chondrocytes also produce latent growth factors and growth factor binding proteins, which help store growth factors in their ECM.  Once cleaved/activated they are able to exert an effect on target cells including hypertrophic chondrocytes (autocrine pathway).

Further details of growth factors and bone morphogenic proteins may be found in WO 03/030873.  Where legally permissible the content of this document is incorporated herein by reference.

Marrow stromal cells (MSCs), also known as mesenchymal stem cells or colony-forming units fibroblastic (CFU-f), are non-haematopoietic multipotent cells that have the ability to adhere to tissue culture plasticware. They were first described by Friedenstein et al., in 1976, who showed that the cells, when cultured in the conditions, adhered to tissue culture plates, growing as a clonal foci with fibroblast morphology. The CFU-f are indicative of the number of CFU in the original cell suspension. Rat BMSC in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate, differentiate into osteoblast-like cells which express alkaline phosphatase (APase), synthesise osteocalcin, and lay down an extracellular matrix which calcifies and has ultra-structural properties similar to bone (Rickard et al., 1994; Maniatopoulos et al., 1988; Satomura & Nagamaya, 1991; Scutt & Bertram, 1995). Thus, the positive staining colonies for APase, calcium, and, collagen, suggests the presence of osteoblast-like cells.

Osteoinduction or fracture repair is a sequential cascade whereby mesenchymal cells are recruited and undergo cell division, followed by differentiation into cartilage, and then replacement of the cartilage by bone. Since cartilage is formed prior to bone,

BMPs can also be termed cartilage morphogenetic proteins. The process of fracture repair involves a complex interaction of many local and systemic regulatory factors. However, the role of BMPs in this process is not fully understood.

## Background to Downstream Processing by γ-Irradiation

The manufacture of biopharmaceutical products using mammalian cell lines is a relatively new technology that allows for the creation of a multitude of pharmaceuticals for the treatment of a diverse range of diseases. However, there is a potential risk of contamination of the product with foreign agents derived from the host cell line, raw materials, or, during processing.

Downstream processing of biopharmaceutical products is essential in ensuring the safety of such biological products. Effective downstream processing should isolate the product from the raw materials (cell culture medium etc), and reliably separate the product from contaminants. Regulatory authorities require the incorporation of multiple processes for the inactivation and removal of pathogens that may be present in the product.

There are a number of downstream processing steps that are available for the inactivation and/or removal of pathogens. The techniques currently used for terminal sterilisation include, ethylene oxide, autoclaving, UV-irradiation, and, γ-irradiation. The technique of γ-irradiation is routinely used for the sterilisation of medical devices (Lee et al., 1996), tissue culture sera (Keathley et al., 1993), and foodstuffs (Truswell, 1987), and has been investigated for the sterilisation of pharmaceuticals (Yaman, 2001; Muszynski et al., 2002; Valvo et al., 1999).

γ-Irradiation has been investigated for the sterilisation of human cortical bone transplants, which showed that a dose of 34 kGy was necessary for inactivation of bovine parvovirus (BPV) in frozen bone transplants (Pruss et al., 2002). The bone-derived allograft material which was treated with gamma radiation by Pruss et al was prepared by a process for diaphysis transplants including the following steps:

Preparation of diaphyses from human femurs after removal of all attached muscles and connective tissue from the bone surface;

sawing of diaphyses into segments of 75mm length;

and washing of the bone marrow canal several times with physiological salt solution to remove blood from the tissue.

None of these techniques involved irradiation of a complex mixture of biological material in the form of a cellular extract.

The sensitivity of pathogens to sterilisation by γ-irradiation is due largely to direct damage of genetic material, although the generation of free-radicals accounts for a significant proportion of the sensitivity (Grieb et al., 2002). Work has been published demonstrating that the recovery of biological activity after sterilisation by γ-irradiation at doses sufficient to inactivate bacteria and viruses (15 kGy and 45 kGy respectively) can be beneficially modulated by the incorporation of free-radical scavengers (ascorbate) within the product; lyophilisation of the product to generate fewer free-radicals; and, low temperature or freezing of the product before sterilisation (Grieb et al., 2002).

Grieb et al tested the effective use of gamma irradiation for pathogen inactivation on a pure sample of a biological protein. The chosen protein was insulin monoclonal antibody (IgG) from a commercial source. Irradiation was carried out to a total dose of either 15 or 45kGy and the irradiations were carried out at 4°C. In one experiment a freeze dried sample of IgG protein was irradiated in the presence of 200mM ascorbate and subsequently protein recovery was demonstrated. In another experiment insulin monoclonal antibody solutions (not lyophilised proteins) in the presence of 200mM ascorbate were spiked with about 9 logs of porcine parvovirus (PPV) infectivity. The samples were irradiated at the chosen two doses and viral inactivation was demonstrated. This work indicated that pure samples of individual biological proteins could be irradiated in lyophilised form and biological activity of the protein could be recovered. This work also indicated that solutions of the individual protein could be irradiated in order to achieve viral inactivation whilst maintaining biological activity.

The experiments of Grieb et al did not involve irradiation of a complex mixture of biological materials in the form of a cellular extract.

A major problem in producing biological materials for patient therapy is defining a production process that is Regulatory Friendly.

Biologicals, like BMP-2 and OP-1 (BMP-7), are generally provided as purified molecules for clinical use. However, ECM is a cell-derived biological material comprising numerous growth factors, cytokines and matrix proteins that act in concert to induce new bone formation. This osteoinductive activity of ECM derives from the complex itself; it is not associated solely with one component of the complex like BMP-2.

The artisan knows that living organisms and the biological activity of individual proteins are highly sensitive to radiation. This is why irradiation is used as a sterilizing process – to destroy contaminating viruses and bacteria, and knockout the biological activity of proteins, for example.

Since the osteoinductive activity of ECM derives from a variety of biologically active ingredients within it, the man skilled in the art would assume that its osteoinductive ability would be destroyed after treatment with irradiation. After all, the greater the complexity of the mix, and the greater the requirement for that mix for osteoinductivity, the more likely it would be for the activity of ECM to be destroyed by irradiation.

WO 96/14400 provides a method for producing human cell lines. The method uses precursor or undifferentiated cells treated with an immortalising agent which is susceptible to environmental conditions in order to provide conditionally immortalised precursor or undifferentiated cells. Selective activation/deactivation of the immortalising agent is followed by selective activation of differentiation to produce cell lines exhibiting a chosen phenotype. This method may be used to produce immortalised hypertrophic chondrocyte-like cells which may be used in the present invention. Where legally permissible the content of this document is incorporated herein by reference.

WO 96/18728 provides chondrocytes and chondrocyte cell lines. In one aspect the document provides human hypertrophic chondrocyte cell lines, methods for producing human hypertrophic chondrocyte cell lines, and a method of producing ECM from said hypertrophic chondrocyte cell lines for use in repair and/or replacement of damaged tissue. The document discloses new biomaterials for use in replacing skeletal tissues such and hips and joints. Cell lines produced according to the teaching of this document may be used in the present invention. Where legally permissible the content of this document is incorporated herein by reference.

WO 03/030873 entitled "Therapeutic Biological Product and Method for Formation of New Vascularised Bone" provides an extracellular material in freeze-dried form obtained from skeletal cells and which has osseoinductive (osteoinductive) activity. This activity may be used in bone repair and regeneration. This document also describes methods for producing osseoinductive extracellular material, compositions containing the material as active ingredient, and methods of treatment using the material. The lyophilised ECM described in this document may be used in the present invention. Where legally permissible the content of this document is incorporated herein by reference. Note that the terms "osseoinductive" and osteoinductive" are equivalent and may be used interchangeably.

BRIEF DESCRIPTION OF THE INVENTION

The present invention provides a method for producing osseoinductive extracellular material from skeletal cells which method comprises or consists of the steps of:

    1.     culturing skeletal cells in a suitable culture medium;

    2.     harvesting extracellular material produced by said cultured cells;

and optionally isolating and/or purifying said harvested materials;

3.  optionally lyophilising said material; and

4.  irradiating said material with gamma radiation.

The method preferably involves lyophilisation prior to irradiation. However lyophilisation may follow irradiation.

According to the invention the irradiating step may be carried out on the material which includes a radioprotectant moiety. This moiety may be added prior to irradiation or may be already included in the harvested material (for example by virtue of being an ingredient in the culture medium). The radioprotectant moiety may be a free radical scavenger which acts to reduce the capacity of the irradiation process to generate free radicals. The radioprotectant may additionally or alternatively be an anti-oxidant which acts to reduce or protect against reactive oxygen species which could be formed by the irradiation process. The radiation dose used in the irradiating step according to the invention will be at a level which does not destroy the osseoinductive activity of the material. Preferably, the dose level of irradiation should substantially all of the biological activity of the material to be maintained following the irradiating step. As used herein, the term "substantially all biological activity" means that in a comparative test the irradiated material is therapeutically useful for osseoinduction when compared to non-irradiated material. Osseoinduction may be defined as a process by which undifferentiated cells are caused to form osteoblasts.

The level of irradiation may be chosen in order to achieve the desired aims of irradiation (as described below). In certain circumstances doses as low as 100Gy or as high as 45kGy may be appropriate. Within this range of 100Gy-45kGy the present invention explicitly contemplates using any individual numerical dose within the range, and any sub-range of doses, and such doses and dose ranges constitute the subject matter disclosed herein. In the detailed example described below, the chosen doses are 5, 10, 15 and 20 kGy but these chosen values are not intended to be limiting of the scope of the invention.

The temperature at which irradiation is carried out may be chosen in order to achieve the desired aims of irradiation (as described below). In certain circumstances temperatures as low as -30°C or as high as +80°C may be appropriate. Within this range of -30°C to +80°C the present invention explicitly contemplates using any individual numerical temperature within the range, and any sub-range of temperatures, and such temperatures and temperature ranges constitute the subject matter disclosed herein. In the detailed example described below, the chosen temperature is room temperature but this chosen value is not intended to be limiting of the scope of the invention.

In view of the fact that the extracellular material from skeletal cells which is used in this invention is a complex mixture of active ingredients comprising many different bio-molecules, it is surprising and commercially important that ECM is able to maintain biological activity after gamma irradiation. The irradiation may be at a level that this effective as a microbe-reducing step in a production process. In exploiting the potential therapeutic uses of ECM it is a particular advantage that the invention can provide a means to obtain a complex biological material for patient therapy which can confidently be provided substantially free of contaminating viruses and bacteria.

In a further aspect, the present invention provides a method as described above for producing a purified osseoinductive extracellular material. By "purified" it is meant that this material has, at least, a reduced level of microbial activity compared to the non-irradiated material, and the material may be substantially or completely free of bacteria and/or viruses.

The term "substantially free" means that the product of the method is acceptable to an appropriate Regulatory Authority for use as a therapeutic/medical product, especially such a product for human use or other animal use. For example, this level of purity may be defined by having levels of bacterial activity and/or levels of viral activity at or below officially approved levels.

According to this aspect, the invention also provides an osseoinductive extracellular material substantially free of bacteria and/or viruses.

Although it may be preferred in most circumstances to provide "pure" material, it is to be noted as a feature of the method of the invention that any level of microbe killing can be advantageous in a manufacturing process and can have real value as part of the Quality Assurance, Regulatory and Manufacturing Strategy. It is not essential that the microbe killing is absolute.

Where legally permissible, the present invention includes methods of treatment of humans and non-human animals using the products of the methods described herein.

The terms "bone repair" and "bone regeneration" used herein should be interpreted in their broadest possible sense as including any medical indication or therapeutic process in which osseoinductive activity is desirable. The osseoinductive material may be used in conjunction with any medical device, where the material is to promote/augment supplemental bone formation in, on, or around the device.

The medical indications to which osteoinductive material may be applied include but are not limited to the following: bone fractures; surgical bone loss e.g. resulting from

removal of cancerous bone, craniomaxillofacial surgery and cranioplasty; joint revision including hip, knee, shoulder, and small joint replacements;bone trauma including all orthopaedic and craniomaxillofacial fractures e.g. spinal fusion following laminectomy inclusive of total disc prosthesis and nuclear prosthesis; osteoporetic fractures and bony spinal injury; congenital bone defects e.g. osteogenesis imperfecta; bone structures requiring supplementation such as bone void filling e.g. following a craniotomy; osteoporosis; and periodontal defects such as oral and periodontal repair including the filling of intrabony voids and alveolar ridge augmentation and voids in the jawbone; periodontal repair of alveolar bone and preparation of alveolar bone for implants and prostheses; and supplementation/augmentation of bone formation in combination with prostheses, including joints (hip, knee, ankle, elbow), dental implants, maxilliofacial devices and spine devices.

The invention will now be described in further detail below, with reference to the accompanying examples and figures. The following detailed description is illustrative of the invention and is not intended to be limiting to the scope of the invention. The scope of the invention is defined by the claims attached hereto.

In particular, the following detailed description of the invention utilises the applicant's proprietary product referred to as "Skeletex$^{TM}$". This product is described in WO 03/030873 and may be produced using techniques described in WO 96/14400 and WO 96/18728. It will be apparent to those skilled in the art that other hypertrophic chondrocyte-like cells could be produced and could be used as the source of ECM for use in the present invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows photographs demonstrating the effects of gamma radiation on Skeletex$^{TM}$-induced total colony formation of rat bone marrow stromal cells. Treatments were renewed on days 6 and 8. The experiment was stopped after 12 days.

Figure 2 shows photographs demonstrating the effects of gamma radiation on Skeletex$^{TM}$-induced alkaline phosphatase-positive colony formation of rat bone marrow stromal cells. Treatments were renewed on days 6 and 8. The experiment was stopped after 12 days.

Figure 3 shows photographs demonstrating the effects of gamma radiation on Skeletex$^{TM}$-induced calcium-positive colony formation of rat bone marrow stromal cells. Treatments were renewed on days 6 and 8. The experiment was stopped after 12 days.

Figure 4 shows photographs demonstrating the effects of gamma radiation on Skeletex™-induced collagen-positive colony formation of rat bone marrow stromal cells. Treatments were renewed on days 6 and 8. The experiment was stopped after 12 days.

## DETAILED DESCRIPTION OF THE INVENTION

## MATERIALS

### Test Articles

Skeletex™ was harvested from confluent HFF Cl 3 cells, passage 43 (2 x T500) on 07/12/2001; passage 44 (8 x T500) on 10/12/2001); and from non-confluent HFF Cl 3 cells, passage 45 (2 x T500) on 07/12/2001; passage 44 (2 x T500) on 07/12/2001; passage 43 (2 x T500) on 07/12/2001; passage 43 (2 x T500) on 10/12/2001; passage 44 (2 x T500) on 10/12/2001, and passage 45 (2 x T500) on 10/12/2001 The Skeletex™ material was stored at -80°C until lyophilisation on 11/03/2002. Lyophilisation yielded 66.5 mg of Skeletex™ which was aliquoted into 15 x 2 ml Wheaton '320' amber glass vials for γ-irradiation.

### Reference and Control Articles

Prostaglandin $E_2$: Sigma, Cat. N° P-4172; Lot 41K1470.

### Equipment

| Description | Model / Product ID code |
|---|---|
| +4°C Fridge/-20°C Freezer, Philips | Compact Combi; Dept code OP240 |
| -80°C Freezer | Sanyo Ultralow |
| Balance | Precisa 125A |
| Centrifuge, Harrier 18/80 refrigerated | Serial number SG99/12/052 |
| Digital camera | Sony MVC-FD88 |
| Haemocytometer | ? |
| Hypodermics | 21G , Becton Dickinson Microlance 3, Cat. No 2003-02 |
| Incubators | ICN Flow, serial number 9307-001, LEEC GA2010 |
| Laminar Flow Hood | Biomat Class II, Dept. code, X219/FC8 |

| Microcentrifuge | MicroCentaur, MSE, Sanyo, Serial number SG93/03/873 |
| Microscope | Bausch & Lomb |
| Pipette fillers, | Pipetboy acu. Ser. N°s. 200065, 100593 |
| Pipettes | Gilson P10 – P1000 |
| Pipettes | Eppendorf P2.5 – P5000 |
| Water bath, Techne | Tempette TE-8A; Dept code OP276 |

## Plasticware and Disposables

| Material | Product ID/code | Supplier |
| --- | --- | --- |
| 0.2μm syringe filters | Cat N°. 90-2520 | Nalgene |
| 1.5 ml Eppendorf tubes | Cat. N° 72.690 | Sarstedt |
| 10 ml stripettes | Cat N° 47110 | Costar |
| 30ml Universal containers | Cat N° 128A | Sterilin |
| 5 ml stripettes | Cat N° 4051 | Costar |
| Material | Product ID/code | Supplier |
| 50 ml stripettes | Cat N° L0150-1 | Costar |
| Cloning cylinders | C7983-50EA | Sigma |
| Disposable pipette tips 1000 | Cat N°. 2069E | Fisher |
| Disposable pipette tips 100E | Cat N° 2065E | Fisher |
| Disposable pipette tips 200 | Cat N°. 2069 | Fisher |
| Syringes | Cat N°. 300185 | Beckton-Dickinson |
| T12 flasks | Cat N° 353107 | Falcon |
| T500 flasks | Cat N°132913 | Nunc |
| Wheaton'320' amber glass vials. 2 ml. | VGA-825-010U | Fisher |

## Reagents

| Material | Product ID/code | Supplier |
| --- | --- | --- |
| Alazarin red | Cat. N° A-5533 | Sigma |
| L-Ascorbic acid 2-phosphate | Cat. N° A-8960 | Sigma |
| Boric acid | Cat. N° B-6768, lot | Sigma |

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|---|---|---|
| Dexamethasone | Cat. N° D-8893 | Sigma |
| Dulbecco's Modified Eagle Medium (DMEM) (1X) | Cat. N° 10938-025, Lot 3062294 | Invitrogen |
| Dulbecco's Phosphate Buffered Saline (PBS) | Cat N° 14190-094, Lot 3061707 | Invitrogen Ltd |
| Fast Red | Cat. N° F-2768; Lot 89H5017 | Sigma |
| Foetal bovine serum (FBS) (Australia sourced) | Cat N°. 10099-141, Lot 2822760S | Invitrogen Ltd |
| L-Glutamine, 200mM | Cat N° 25030-024, Lots 3062308, 3061811 | Invitrogen Ltd |
| $\beta$-Glycerophosphate | Cat. N° G-9891 | Sigma |
| Hydrochloric acid | Cat. N° 101252F | BDH |
| MEM alpha medium (with) L-glutamine, ribonucleosides, deoxyribonucleosides) | Cat no. 22571-020, Lots 3059737 | Invitrogen Ltd |
| Methylene blue | Cat. No 52015 | Raymond Lamb |
| Naphthol phosphate | Cat. N° N-2250; Lot 28H5237 | Sigma |
| Non -essential amino acids (NEAA) without L-glutamine | Cat N° 11140-035, Lot 3059965 | Invitrogen Ltd |
| Penicillin/Streptomycin (P/S) | Cat N° 15140-122, lot 1106810 | Invitrogen Ltd |
| Perchloric acid | Cat. N° 294582X, lot B506572 | BDH |
| Picric acid | Cat. N° 681400 | Searle |
| Prostaglandin $E_2$ | Cat. N° P-4172, lot 41K1470 | Sigma |
| Rat osteocalcin EIA kit | Cat. N° BT-490 | Biomedical Technologies Inc |
| Sirius red | Cat. No 35780 | Raymond Lamb |
| Sodium hydroxide | Cat. N° 307314P, lot K23299650 | BDH |
| Sodium Pyruvate MEM 100 mM | Cat. N° 11360-039; lot | Invitrogen |

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|---|---|---|
| Trizma hydrochloride | T-7149 | Sigma |
| Trypan blue stain 0.4% | Cat. N° 15250-061, lot 1134142 | Invitrogen |
| Trypsin/EDTA | Cat N° 25300-062, lot 3061160. | Invitrogen |

## METHODS AND PROCEDURES

### Preparation of Culture Media and Ancillaries

### Foetal Bovine Serum

FBS (500ml bottles) was received and stored at -20°C in room E03. Bottles of serum were thawed at room temperature and heat-inactivated by placing in a water bath at 56°C for 30 minutes. These were then aliquoted aseptically in the laminar flow hood, into 25ml volumes in universal tubes and stored at -20°C in room E03.

### HFF Cl 3 Growth medium (500 ml solution)

MEM alpha medium (1X) (440 ml) was supplemented with heat-inactivated foetal bovine serum (FBS) (10% v/v), l-glutamine (2 mM), 100X MEM non-essential amino acids (NEAA) (1%). After preparation, the complete growth medium was stored at 4 °C in Lab E03 with an expiry of two weeks. Sterility testing was not performed routinely.

### Osteogenic Growth Medium (500 ml solution)

DMEM (1X) (415 ml) was supplemented with heat-inactivated foetal bovine serum (12% v/v), l-glutamine (2 mM), sodium pyruvate (1 mM), penicillin/streptomycin (1% v/v), β-glycerophosphate (10 mM), l-ascorbic acid 2-phosphate (50 µg/ml), and dexamethasone (10 nM). After preparation, the complete growth medium was stored at 4 °C in Lab E03 with an expiry of two weeks. Sterility testing was not performed routinely.

### Harvesting of Skeletex™

A Skeletex™ is an active extracellular material derived from cultures of immortalised human hypertrophic chondrocyte-like cells. The Skeletex™ material synthesised by HFF Cl 3 cells and secreted into the cell culture growth medium, was harvested from confluent HFF Cl 3 cells, passage 43 (2 x T500) on 07/12/2001; passage 44 (8 x T500) on 10/12/2001); and from non-confluent HFF Cl 3 cells, passage 45 (2 x T500) on 07/12/2001; passage 44 (2 x T500) on 07/12/2001; passage 43 (2 x T500) on 07/12/2001; passage 43 (2 x T500) on 10/12/2001; passage 44 (2 x T500) on 10/12/2001, and passage 45 (2 x T500) on 10/12/2001. The conditioned growth media was transferred aseptically to centrifuge tubes (50 ml) and centrifuged at 250 x g for

5 minutes at room temperature. After centrifugation, the majority of the supernatant was carefully discarded, leaving a fine pellet which contained the active ingredient of Skeletex™. The pelleted Skeletex™ material produced from each harvest was resuspended in the remaining supernatant (typically 100 μl approx per T500 flask), combined and stored in 1.5 ml eppendorf tubes at −80°C.

## Lyophilisation of Skeletex™

Skeletex™ was removed from the -80°C freezer and allowed to thaw at room temperature for 1 hour. Once thawed, aliquots of Skeletex™ were combined in a 30 ml universal container, and re-frozen at -80°C, before being lyophilised under vacuum for 3 hours. The lyophilisation process was performed on 11/03/2002 and yielded 66.5 mg of lyophilised Skeletex™. Lyophilised Skeletex™ was aliquoted into 15 x 2 ml Wheaton '320' amber glass vials (>2 mg of lyophilised Skeletex™/vial).

## γ-Irradiation of Lyophilised Skeletex

Three aliquots of lyophilised Skeletex™ were prepared for each dose of γ-irradiation investigated. Aliquots were dispatched to the Gamma Processing Centre of Isotron at Swindon (UK) for γ-irradiation at 4 doses, 5 kGy, 10 kGy, 15 kGy, and, 20 kGy. Irradiation was carried out at room temperature.

## In Vitro Bioassay

## Isolation of Rat Bone Marrow Cells

A female Wistar rat (120 − 150 g) was sacrificed by cervical dislocation, the fur around the hind legs sprayed with industrial methylated spirit (IMS) to reduce fungal contamination, and the hind legs removed cleanly at the hip joint. Under aseptic conditions, soft tissue was dissected away revealing the leg bones, which were then separated at the knee. The growth plate of the femur was removed, and the bone cut below the hip joint to reveal bone marrow. The bone was placed cut-side down in an eppendorf tube containing an cloning ring, and centrifuged at 2,000 rpm for 2 minutes. After centrifugation, the bone and cloning ring were discarded leaving the pelleted bone marrow. The bone marrow was resuspended in 10 ml of Osteogenic growth medium (Section 5.1.3). A single cell suspension was created by repeatedly drawing through a 21 g needle, and nucleated cells were identified by staining with trypan blue, and counted using a haemocytometer.

## Fibroblastic Colony Forming Unit (CFU-f) Assay

Rat bone marrow cells were seeded at $4 \times 10^4$ cells/cm$^2$ in T12 flasks in Osteogenic growth medium (3 ml), containing duplicate treatments of: control (untreated) cells, PGE$_2$ (100 nM), Skeletex™ (1 mg lyophilised; non-irradiated), Skeletex™ (1 mg

lyophilised; 5 kGy irradiated), Skeletex™ (1 mg lyophilised; 10 kGy irradiated), Skeletex™ (1 mg lyophilised; 15 kGy irradiated), Skeletex™ (1 mg lyophilised; 20 kGy irradiated). The Osteogenic medium was changed after 6 days and 8 days, with treatments being renewed at each media change. The cultures were maintained for 12 days after which, cells were washed with PBS and then fixed for 10 minutes by the addition of ethanol (100%) before sequentially staining for the osteoblastic markers alkaline phosphatase, calcium, collagen, and total colony formation.

## Alkaline Phosphatase Staining of CFU-f Cultures

Fixed CFU-f cultures were stained for alkaline phosphatase (APase) by incubating for 30 minutes at room temperature in 3 ml of Tris HCl (20 mM, pH 8.5) containing naphthol phosphate (50 μg/ml) and fast red (1 mg/ml). Colonies were then washed under running tap water until all excess dye had eluted, and allowed to dry before taking a digital image. Colonies were destained by incubating in IMS overnight.

## Calcium Staining of CFU-f Cultures

Fixed CFU-f cultures were stained for calcium with 3 ml of alazarin red (1 mg/ml, pH 5.5) by shaking at room temperature for 30 minutes. Excess stain was removed by washing under running tap water. Plates were allowed to dry before taking a digital image of the calcium-positive (cfu-f$_{Ca}$) colonies. Mineralised colonies were destained by addition of 3 ml of 5% perchloric acid, and then washed with tap water.

## Collagen Staining of CFU-f Cultures

Collagen-positive (cfu-f$_{col}$) colonies were determined by a method described by Lopez-de-Leon & Rojkind (1985). Briefly, after destaining mineralised colonies, cultures were stained with 3 ml of sirius red (1 mg/ml) in saturated picric acid by shaking at room temperature for 18 hours. Excess stain was removed by washing under running tap water. Cfu-f$_{col}$ cultures were allowed to dry before taking a digital image of the collagen-positive colonies. Colonies were destained by shaking in 0.2 M sodium hydroxide/methanol (50:50 v/v), and then washing in tap water.

## Total Colony Staining of CFU-f Cultures

Total colony numbers were determined by staining with 3 ml methylene blue (1 mg/ml) in borate buffer (10 mM, pH 8.8) for 30 minutes at room temperature. Excess stain was removed by washing under tap water. Plates were allowed to dry before taking a digital image.

## RESULTS

**Effect of γ-Irradiation on the Osteoinductive Efficacy of Lyophilised Skeletex™**

The effect of γ-irradiation on the osteoinductive efficacy of lyophilised Skeletex™ (1 mg) from HFF Cl 3 cells was investigated. The osteoinductive effect of lyophilised Skeletex™ material was compared with control (untreated) cells, with the bone anabolic agent $PGE_2$ (100 nM)-treated cells (positive control), and with lyophilised Skeletex™ that had been γ-irradiated at various dose levels (5 kGy, 10 kGy, 15 kGy, and, 20 kGy).

The results of the total colony formation staining (Figure 1) show that lyophilised Skeletex™ (1 mg) induces the formation of a greater number of colonies than control (untreated) or $PGE_2$ (100 nM)-treated cells. The total number of colonies induced by · lyophilised Skeletex™ was not effected by γ-irradiation at any of the doses investigated (5 – 20 kGy).

Staining for alkaline phosphatase (Figure 2) reveals that a large proportion of the colonies induced by lyophilised Skeletex™ are positive for alkaline phosphatase. The number of alkaline phosphatase-positive colonies induced by lyophilised Skeletex™ is much greater than either control (untreated), or $PGE_2$ (100 nM)-treated rat marrow stromal cells. Treatment with γ-irradiated Skeletex™ at the doses investigated, did not effect the number of alkaline phosphatase-positive colonies when compared with Skeletex™ that had not been γ-irradiated.

The results of the calcium stain (Figure 3) show that treatment with lyophilised Skeletex™ induced rat marrow stromal cells to elaborate an extracellular matrix (ECM) that was subsequently mineralised. The number of mineralised colonies that were induced by lyophilised Skeletex™ is greater than either control (untreated), or $PGE_2$ (100 nM)-treated rat marrow stromal cells. γ-Irradiation of Skeletex™ at the dose levels investigated, did not effect the mineralisation of colonies when compared with non-irradiated lyophilised Skeletex™.

Collagen staining (Figure 4) reveals that lyophilised Skeletex™ induced a large proportion of the colonies formed to stain positive for collagen. The number of collagen-positive colonies induced by treatment with lyophilised Skeletex™, was greater than either control (untreated), or $PGE_2$ (100 nM)-treated rat marrow stromal cells. γ-Irradiation, at the doses investigated, did not alter the formation of collagen-positive colonies induced by Skeletex™.

## CONCLUSION

These results show that downstream processing by γ-irradiation (5 – 20 kGy) did not effect the osteoinductive efficacy of lyophilised Skeletex™ in an *in vitro* model. The results from the qualitative stains for makers of osteoblastic differentiation (alkaline phosphatase, calcium, and, collagen) show that colony formation induced by lyophilised Skeletex™ was not modulated by γ-irradiation at the dose levels investigated.

Lyophilisation of the Skeletex™, as well as the presence of 250 µM ascorbate in the lyophilate, preserved activity during the irradiation process.

In conclusion, this data reveals that γ-irradiation at doses up to 20 kGy does not effect the osteoinductive efficacy of Skeletex™ at a single dose (1 mg) in an *in vitro* model.

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## GLOSSARY

| DMEM | Dulbecco's Modified Eagle Medium |
|------|----------------------------------|
| °C | degree centigrade |
| FBS | foetal bovine serum |
| ml | millilitre |
| mM | millimolar |
| ng | nanogram |
| nM | nanomolar |
| pg | picogram |
| PPV | porcine parvovirus |
| µg | microgram |
| µl | microlitre |
| µm | micrometre |
| µM | micromolar |